

Murine Cytomegalovirus Infection-Induced Polyclonal B Cell Activation Is Independent of CD4⁺ T Cells and CD40

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The results of this study demonstrate that murine cytomegalovirus (MCMV) induces polyclonal B cell activation in mice during the acute phase of primary infection. First, flow cytometric analysis revealed that surface expression of CD45R, IgM, and Ig κ by splenocytes from MCMV-infected mice was significantly reduced with a concomitant increase in the frequency of surface IgG-expressing cells. Second, ELISPOT assays demonstrated that the changes revealed by flow cytometry were paralleled by increases in the numbers of IgG-producing cells, especially those secreting IgG_{2a}. Third, the IgG antibodies from MCMV-infected animals reacted against a variety of self and foreign antigens. MCMV-induced B cell activation was independent of CD4⁺ T-cell-mediated help and CD40, since activation was observed in two models of mice deficient for this T cell subset and in mice deficient for CD40. Reverse transcription–polymerase chain reaction analysis showed that mRNA transcripts for the cytokines IL-6, IL-10, and IFN- γ were rapidly induced following infection with MCMV, but only IL-6 and IFN- γ proteins were detectable by ELISA. In addition, the numbers of cells producing IL-6 and IFN- γ were significantly increased in the spleen. The magnitude of the polyclonal B cell activation response was diminished by 50% in IL-6-deficient mice but not in mice lacking IFN- γ . In the absence of IFN- γ , surface expression and serum levels of IgG_{2a} were reduced while IgG₁ expression was increased. © 1998 Academic Press

INTRODUCTION

The high incidence of human cytomegalovirus infection in the general population is seldom a major medical problem, but in immunocompromised patients, it is a leading cause of mortality (Neiman *et al.*, 1973; Moskowitz *et al.*, 1985; Ho, 1991a). Because of several similarities in its biology and disease spectrum, murine CMV (MCMV) has been a useful model in understanding the pathogenesis of CMV infections in humans (Hudson, 1979). Like other herpesvirus infections, acute CMV infection is followed by chronic infection and viral latency (Bruggeman, 1993). Reactivation of latent virus can occur under conditions of immunosuppression and immunodeficiency (Fiala *et al.*, 1991; Ho, 1991b; Bruggeman, 1993).

Establishment of latency in the infected host by MCMV and other herpes viruses is poorly understood but may be due to the ability to avoid elimination in the acute phase of the disease (Banks and Rouse, 1992). Early in the course of an acute MCMV infection, NK cells are believed to control virus replication prior to the induction of antiviral T cell and antibody responses. During the NK

and T cell responsive phases of the infection, spleen cells from MCMV-infected mice are hyporesponsive to T and B cell mitogens, alloantigens, and viral antigens (Selgrade *et al.*, 1976; Kelsey *et al.*, 1978; Ho, 1980; Loh and Hudson, 1981; Nicholas *et al.*, 1987; Campbell *et al.*, 1989). Despite this suppression of the T cell compartment, an effective antiviral T cell response is nevertheless generated that can control the infection; however, it can prevent neither virus persistence nor the establishment of latency (Koszinowski *et al.*, 1990).

Although passively transferred virus-specific antibodies are protective (Shanley *et al.*, 1981; Farrell and Shellen, 1991), antibodies are not essential for recovery from a primary MCMV infection (Jonjic *et al.*, 1994). Antibodies are important, however, in limiting reactivation and dissemination of latent virus (Jonjic *et al.*, 1994). Infection with HCMV is frequently associated with a number of transient humoral abnormalities—mixed cryoglobulins, cold agglutinins, rheumatoid factor, antinuclear antibodies, and anticomplementary activity (Klemola *et al.*, 1970)—that may be attributable to virus-induced polyclonal B cell activation (Hutt-Fletcher *et al.*, 1983). The appearance of autoantibodies in serum of mice infected with MCMV may also be caused by widespread activation of B cells (Price *et al.*, 1993).

Our preliminary studies of mice injected with MCMV

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strengthened this suggestion in revealing that the transient splenomegaly observed in mice infected as adults was associated with a major expansion of activated B cells and isotype switching from IgM to IgG expression. The experiments described here were designed, first, to determine whether these B cell responses were polyclonal and if they were dependent on the presence of CD4⁺ T cells. Ig synthesis and class switching by B cells responding to T-dependent antigens require cognate T-B interactions through CD40/CD40 ligand (CD40L) binding and cytokines produced primarily by CD4⁺ T cells. Some cytokines affecting B cell physiology can be produced by non-T cells, however, and viral infections are often potent inducers of cytokine expression (Bowen *et al.*, 1992). Further experiments were undertaken to examine the pattern of cytokine expression associated with B cell activation during the course of MCMV infection. The contribution of some of these cytokines and the requirement for CD40 in the B cell activation process were also investigated.

MATERIALS AND METHODS

Mice

Female, specific-pathogen-free (SPF), euthymic C×B-7/ByJ (C×BK) (H-2^b), C57BL/6J (B6) (H-2^b), BALB/cJ (BALB/c) (H-2^d), and B10.BR (H-2^k) mice and B6 and BALB/c mice bearing the nude mutation (*nu*) (B6-*nu/nu*; BALB/c-*nu/nu*) were obtained from the Jackson Laboratories (Bar Harbor, ME) and used at 6 to 16 weeks of age. Mice deficient in MHC class II expression resulting from germline disruption of the *A_B*^b gene (C2D^{-/-}) (Grusby *et al.*, 1991) were fifth generation backcross to B6 mice and were purchased from GenPharm International (Mountain View, CA) along with controls expressing MHC class II (C2D^{+/+}). C2D^{-/-} mice lack CD4⁺ T cells and have increased frequencies of CD8⁺ T cells (Bowen *et al.*, 1992). Mice deficient in CD40 (Kawabe *et al.*, 1994) and IL-6 (Kopf *et al.*, 1994), both of B6 × 129 background, and mice deficient in IFN- γ (Dalton *et al.*, 1993) on a B6 background were bred under SPF conditions at the Animal Breeding Establishment, John Curtin School of Medical Research.

Virus

The Smith strain of MCMV was salivary gland-passaged and a 10% homogenate prepared in Dulbecco's MEM (DMEM) containing 20% veal infusion broth. Mice were inoculated ip with a dose of virus adjusted appropriately for each strain to provide resistance to lethal challenge; this was approximately 10-fold lower than the LD₅₀. The doses given to individual mouse strains are given in Table 1.

TABLE 1
Mouse Strains and Virus Doses

Mouse strain	LD ₅₀ (PFU)	Dose used (PFU)	Gene knockout mice
C×BK	10 ^{5.9}	10 ^{4.9}	—
C57BL/6	10 ^{6.9}	10 ^{5.9}	IFN- γ ^{-/-}
BALB/c	10 ^{5.9}	10 ^{4.9}	—
B10.BR	>10 ^{7.5}	10 ^{6.9}	—
B6- <i>nu/nu</i>	ND ^a	10 ^{3.9}	—
BALB/c- <i>nu/nu</i>	ND	10 ^{3.4}	—
B6 × 129 ^b	10 ^{7.5}	10 ^{5.9}	CD40 ^{-/-} IL-6 ^{-/-}
C2D	10 ^{7.5}	10 ^{6.5}	MHC class II ^{-/-}

^a ND, not determined.

^b A lower virus dose was used for this particular strain because the IL-6^{-/-} and CD40^{-/-} mice, both on a B6 × 129 background, were more susceptible than wild-type B6 × 129 mice to lethal infection with MCMV.

Cell lines

The murine fibroblast NIH-3T3 cells were maintained in DMEM containing 10% heat-inactivated bovine serum (BS), L-glutamine, and antibiotics.

Flow cytometry

The following fluorochrome-conjugated mAbs to murine cell surface antigens were used for single color analysis: FITC-conjugated anti-IgG₁ (clone G1-6.5), anti-IgG_{2a} (clone R19-15), anti-IgG_{2b} (clone R12-3), anti-IgG₃ (clone R40-82), anti-CD45R (B220; clone 6B2), anti-IgA (clone RMA-02), and biotinylated anti-NK1.1 (clone PK 136) were obtained from PharMingen (San Diego, CA). Anti-IgM (clone LO-MM-9; Zymed Laboratories, South San Francisco, CA), anti-Ig κ -chain (clone MCA152F; Serotec Ltd., Oxford, England), and goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) were also used as FITC-conjugated antibody. Avidin-allophycocyanin was obtained from Molecular Probes (Eugene, OR). Spleen cells were prepared and stained as described previously (Karupiah *et al.*, 1993). For each sample, 20,000 events were collected and analyzed on a FACS 440 flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) at the NIH and on a FACscan flow cytometer (Becton Dickinson Immunocytometry Systems) at the JCSMR.

CD4⁺ T cell depletion *in vivo*

Acute depletion of the CD4⁺ T cell subset *in vivo* was achieved using a mAb to the CD4 antigen (clone GK1.5, rat IgG_{2b}). The mAb was partially purified using saturated ammonium sulfate and the concentration was determined by radial immunodiffusion (The Binding Site Inc., San Diego, CA). Mice were treated with the mAb ip at 1 mg/day/mouse twice before infection (days -3 and -1), 1 day after infection (day 1), and every 4 days thereafter

until termination of the experiment. Efficiency of depletion was assessed by flow cytometric analysis of spleen cells; all treated mice had less than 1% CD4⁺ T cells.

Anticytokine mAb treatment

Immunomodulation studies using neutralizing mAbs to cytokines were undertaken to determine whether IFN- γ , TNF- α , or IL-10 contributed to the activation of B cells in this model. MCMV-infected mice were treated with partially purified ascites containing mAbs to IFN- γ (clone XMG-6; 3×1 mg/mouse/week), TNF- α (clone XT-22/11; 3×0.5 mg/mouse/week), or IL-10 (clones SXC-1 and SXC-2; 4×2 mg/mouse/week). The effect of mAb treatment on MCMV-induced B cell activation was assessed by flow cytometry.

Isolation and purification of mRNA from spleens

Individual spleen samples were homogenized in 1 ml RNAzol (Cinna Biotech, Friendswood, TX), and total RNA was isolated according to the manufacturer's instructions. The RNA was resuspended in 200 μ l diethylpyrocarbonate-treated water containing 1 mM EDTA and quantitated spectrophotometrically.

Reverse transcription-polymerase chain reaction (RT-PCR) for cytokine mRNA detection

The relative quantities of mRNA for the cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p35 and p40 chains), IL-13, IFN- γ , TNF- α , and the housekeeping gene HPRT were determined using a RT-PCR procedure as described elsewhere (Svetic *et al.*, 1991; Wynn *et al.*, 1993). The sense and antisense primers and probes for all cytokines and HPRT have been described previously (Svetic *et al.*, 1991; Wynn *et al.*, 1993). The probes were designed to hybridize to a portion of the amplified segment between the primers, which ensured the identity of the segment amplified.

The PCR reaction conditions had been predetermined and defined for each cytokine primer pair such that a linear relationship between input RNA and final PCR product was obtained (Wynn *et al.*, 1993). Known positive and negative controls were included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated with cDNA or any previous PCR products. After amplification, the DNA was analyzed by electrophoresis, Southern blotting, and hybridization with nonradioactive cytokine-specific probes as described previously (Wynn *et al.*, 1993). For each cytokine, results were normalized for the relative quantity of total mRNA by comparison to HPRT. Values are expressed as fold increase/decrease of mRNA expression in the spleen over that of control, uninfected mice (day 0), which are assigned an arbitrary value of 1 and represent the mean and standard deviation of cyto-

kine mRNA levels in the spleens of three individual mice/group.

Cell preparation for ELISpot assays

Animals were killed by cervical dislocation. Single-cell suspensions were prepared from the spleen, bone marrow, mesenteric lymph nodes, and peritoneal exudate and washed three times in RPMI supplemented with 5% FCS. Cells were counted and adjusted to a concentration appropriate for each experiment.

ELISpot for antibody isotype and antigen specificity

Flat-bottom Immulon I microtiter plates (PGC Scientifics, Gaithersburg, MD) were coated with goat anti-mouse IgG (Southern Biotechnology Associates) for 2 h and blocked with 1% BSA in PBS for 2 h. For antibody specificity, flat-bottom plates were coated with ovalbumin, actin, TNP, ssDNA, myoglobin, or HIV-1 gp 120 (Monestier *et al.*, 1986; Klinman and Steinberg, 1987) and blocked as described. After blocking, plates were washed three times with PBS containing 0.025% Tween and rinsed three times with distilled H₂O. Serial dilutions were performed from an initial cell number of 10^6 /well. Cells were incubated in 150 μ l of RPMI 1640 with 5% FCS for 6–7 h at 37°C in a CO₂ incubator, after which plates were washed as described (Klinman and Steinberg, 1987) and then incubated overnight with a 1:1000 dilution of alkaline phosphatase-labeled anti-IgG, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, or IgM (Southern Biotechnology) in PBS-BSA. Washed plates were developed with an agar solution containing 5-bromo-3-chloroindolyl phosphate solution (Sigma Chemical Company). The number of spots/well was used to determine the number of cells/ 10^6 secreting Ig *in vivo*. The total number of cells producing Ig per organ was then calculated and used to determine the total number of cells secreting per animal (i.e., spleen + bone marrow + lymph nodes + peritoneal exudate). Ratios of secreting cells were calculated using the total number of secreting cells.

Cytokine ELISpot

Millititer HA 96-well filtration plates (Millipore Corp., Bedford, MA) were coated overnight with anti-IL-2 (clone JES6-1A12 diluted 1:50; PharMingen), anti-IL-4 (clone BVD6-24G2 diluted 1:75; Endogen, Boston, MA), anti-IL-6 (clone MP5-2053 diluted 1:75; PharMingen), or anti-IFN- γ (clone R4-6A2 diluted 1:100; Lee Biomolecular, San Diego, CA) in 0.1 M carbonate buffer. Plates were blocked with 5% BSA + 0.025% Tween + PBS for 2 h and washed as described. Cells were serially diluted in medium supplemented with 5% FCS as described and incubated in a total volume of 100–150 μ l for 5–6 h. Following incubation, plates were washed with H₂O + 0.025% Tween with a Titertek plate washer (ICN-Flow, Costa Mesa, CA). Anti-IL-2 (clone JES6-5H4 diluted 1:500;

PharMingen), anti-IL-4 (clone IIBII diluted 1:4000), anti-IL-6 (clone MP5-32C11 diluted 1:1000; PharMingen), or anti-IFN- γ (clone XMG1.2 diluted 1:1000; PharMingen) in PBS + 0.025% Tween + BSA was added to the corresponding plates and refrigerated overnight. After incubation for 1 h at room temperature, a 1:2000 dilution of avidin (Vector Laboratories, Burlingame, CA) was added to the washed plates. Plates were then incubated 1 h at room temperature, washed, and developed with BCIP-NBT (Kirkegaard and Perry, Gaithersburg, MD) for 30 min. After developing, plates were rinsed with distilled H₂O and allowed to dry. Spots were counted the following day with a dissecting microscope. Cell numbers were calculated and analyzed as described above.

Detection of antibodies by ELISA

Flat-bottom microtiter plates were coated with ovalbumin, TNP, and myoglobin and blocked as described (Klinman and Steinberg, 1986, 1987). Sera (diluted to 1:50 in PBS-Tween followed by serial twofold dilutions) from uninfected control and MCMV-infected mice were added to wells, and the presence of antibodies reactive with ovalbumin, TNP, and myosin was detected using, in order, biotinylated goat anti-mouse IgM or IgG followed by streptavidin-conjugated alkaline phosphatase and phosphatase substrate. Total IgM and IgG concentrations were determined using appropriate primary goat anti-mouse antibody for capture and biotinylated goat anti-mouse for detection (Southern Biotechnology).

Responses to concanavalin A (Con A) and LPS

Splenocytes (1×10^7) from groups of three uninfected and MCMV-infected mice were cultured in wells of 24-cluster plates in RPMI containing 10% FCS, 500 μ M 2-ME, L-glutamine, sodium pyruvate, and nonessential amino acids (complete RPMI). The T cell mitogen Con A (4 μ g/ml) and B cell mitogen bacterial lipopolysaccharide (LPS; 10 μ g/ml) were added to some cultures, whereas others were left untreated. After 72 h, cultures were harvested and spun at 2000 rpm in a bench-top centrifuge maintained at 4°C for 5 min, the cell-free supernatants were collected, and the presence of cytokines was determined by ELISA.

ELISAs for cytokines

Standard sandwich ELISAs were performed for detection of IL-2, IL-4, IL-10, and IFN- γ in culture supernatants using appropriate primary mAb for capture and biotinylated mAb for detection. Antibody pairs and cytokine standards were obtained from PharMingen. When necessary, supernatants were diluted in complete RPMI before cytokine levels were quantitated. IL-6 in culture supernatants was quantitated using an ELISA kit (Endogen) according to the manufacturer's instructions.

Histopathology

Mice were necropsied, and spleen, liver, lung, salivary glands, and cervical lymph nodes were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin.

RESULTS

CD4⁺ T-cell-independent B cell activation following MCMV infection

Spleen cells from uninfected and MCMV-infected CXBK mice were stained with fluorochrome-conjugated mAbs. Alterations in splenocyte populations were assessed by flow cytometric analysis (Fig. 1). The most obvious changes noted for B cells of infected mice were severely reduced levels of CD45R (B220) and surface IgM and Ig κ expression with concomitant increases in surface IgG expression (Fig. 1A) and cell size (data not shown), consistent with B cell activation. There was also a dramatic increase in the expression of the NK cell marker NK1.1 (Fig. 1). Changes in T cells were more modest. The frequency of CD4⁺ cells was reduced from 17% in normal spleens to 12% in spleens of mice infected for 10 days, while the frequencies of CD8⁺ T cells were 7 and 6%, respectively, for these animals. These changes correlated with increases in spleen weight and cellularity and were apparent as early as 5 days after infection (data not shown). Maximal changes were detected at 10 days (Fig. 1A), but levels had returned to normal by 14 days after infection (data not shown). Very similar changes were observed following infection of another MCMV-susceptible mouse strain (BALB/c) and two genetically resistant mouse strains (B10.BR and B6), the latter strains requiring infection with higher doses of virus (see Materials and Methods). In both resistant and sensitive strains, B cell activation, assessed by flow cytometry, was evident provided the mice were productively infected with an appropriate dose of virus.

We next determined whether this phenomenon of B cell activation was dependent on CD4⁺ T cells. Flow cytometric analysis of splenocytes from MCMV-infected CXBK mice depleted of CD4⁺ T cells by treatment with mAb GK1.5 established that B cell activation in these circumstances did not require the presence of this subset of T cells. Although the effects of infection were slightly diminished compared with those seen in control MCMV-infected mice (Fig. 1A), B cells from CD4⁺ T-cell-deficient mice clearly had reduced levels of CD45R (B220), IgM, and Ig κ , concomitant with an increase in the frequency of surface IgG-expressing cells, consistent with activation (Fig. 1B). Similar observations were made in mice lacking CD4⁺ cells because of disruption of the A β gene (C2D^{-/-}) and in two strains (B6, BALB/c) of

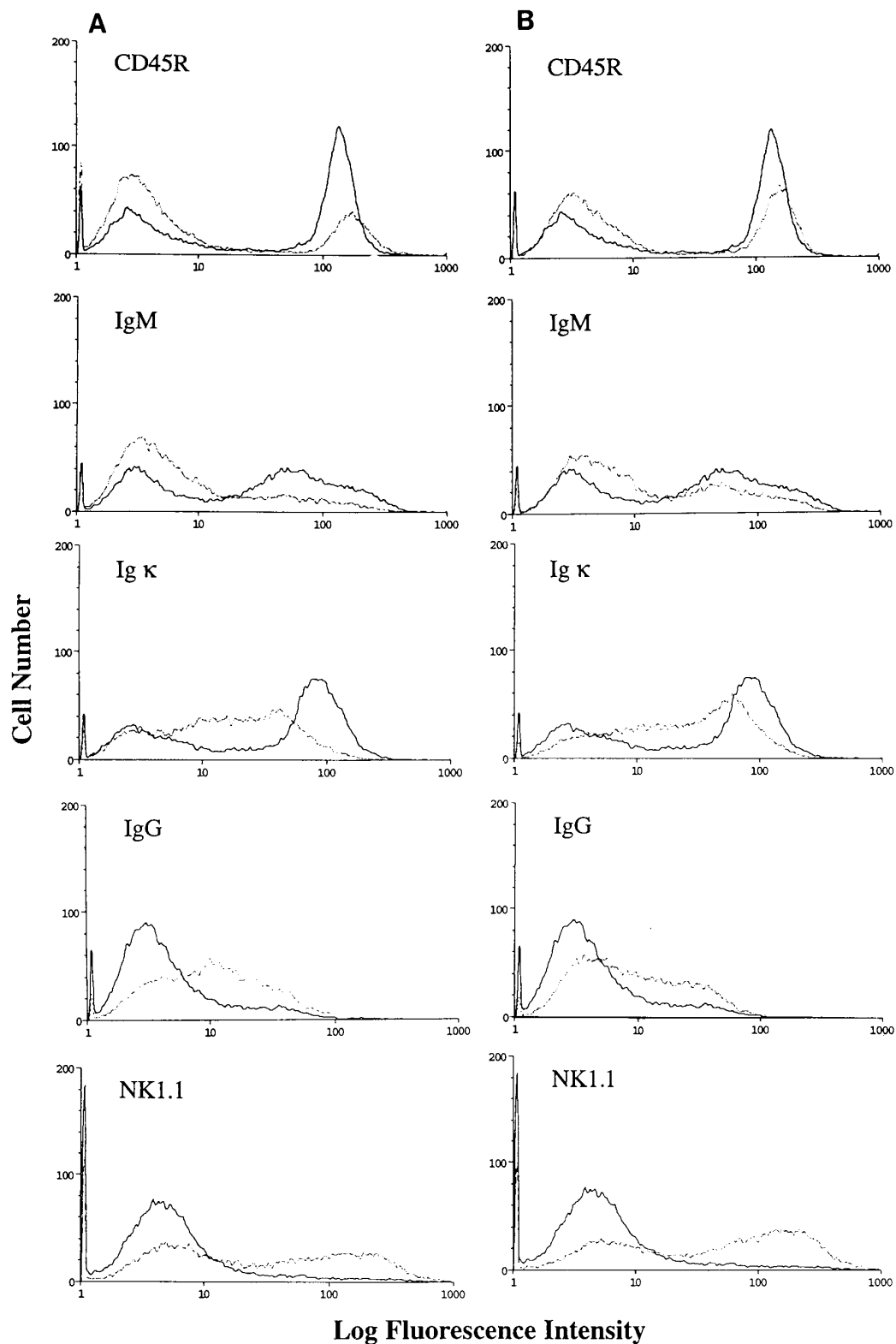


FIG. 1. Flow cytometric analysis of splenocytes for expression of B cell markers and NK1.1 antigen. Spleens were obtained from control, uninfected (solid lines), or 10-day MCMV-infected (dashed lines) CXBK mice. (A) Expression of CD45R, IgM, Ig κ , IgG, and NK1.1 antigens on splenocytes of CXBK mice with intact CD4⁺ T cells. (B) Expression of the same markers on splenocytes of mice depleted of CD4⁺ T cells. Data shown are representative of two separate experiments with five mice in each group.

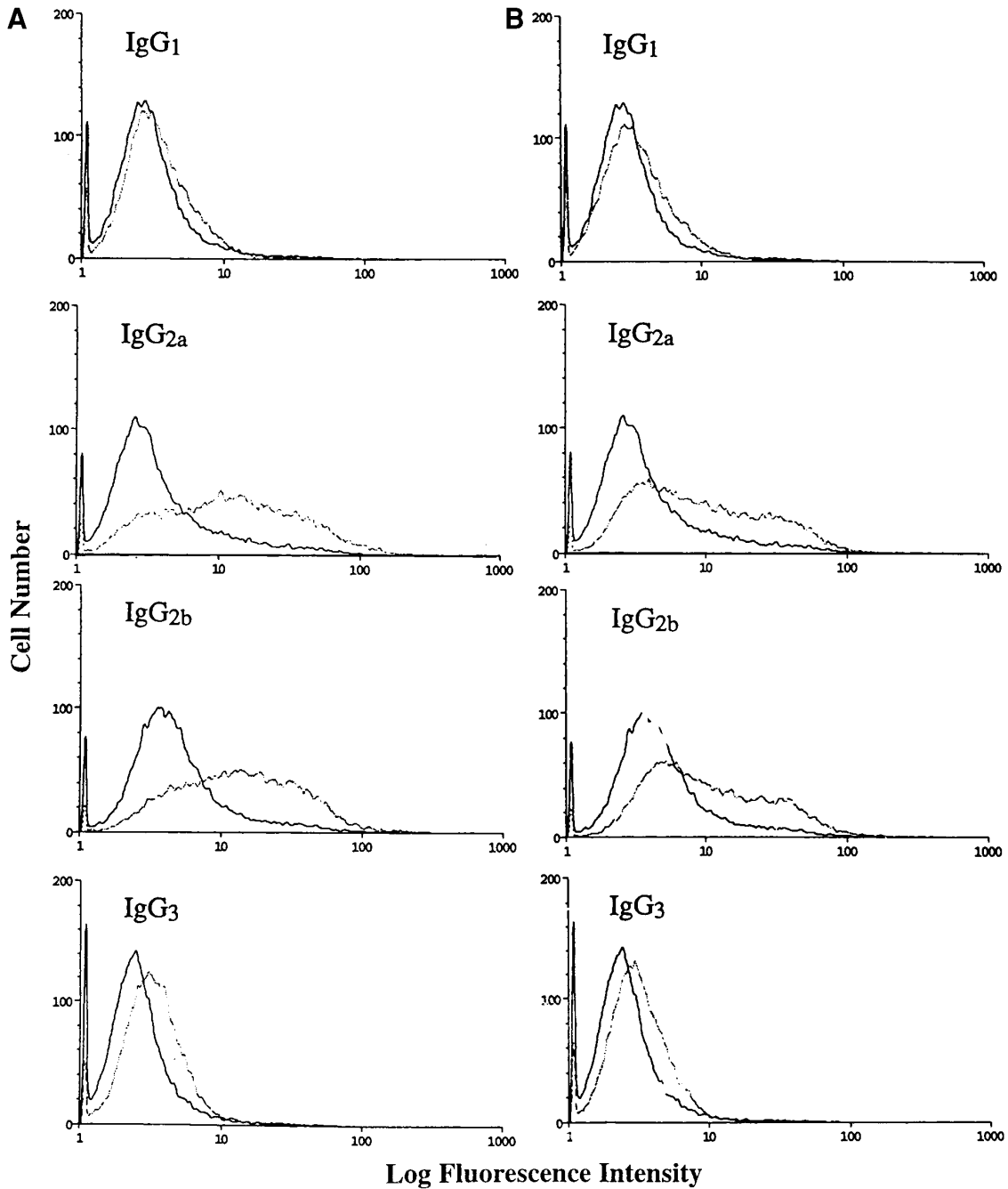


FIG. 2. Analysis by flow cytometry for surface expression of IgG subclasses using the same spleen cell samples described in Fig. 1. IgG subclass expression on splenocytes from control, uninfected (solid lines), or MCMV-infected (dashed lines) CXBK mice with intact CD4⁺ T cells (A) and in mice depleted of CD4⁺ T cells (B). Results shown are representative of two separate experiments with four mice in each group.

athymic (*nu/nu*) mice that lack functional T cells (data not shown).

Further analysis using IgG subclass-specific mAb conjugates indicated pronounced increases in surface expression of IgG_{2a} and IgG_{2b} and marginal increases in surface IgG₁ and IgG₃ expression on spleen cells from MCMV-infected CXBK mice (Fig. 2A). These changes were also evident in CD4⁺ T-cell-depleted CXBK mice (Fig. 2B) and in the CD4⁺ T-cell-deficient C2D^{-/-} mice (data not shown).

Enumeration of antibody-producing cells by ELISpot assay

In order to establish whether the increased levels of surface IgG expression on B cells were paralleled by increases in the number of antibody-producing cells, we used an ELISpot assay to enumerate isotype-specific and IgG subclass-specific antibody-producing cells using lymphocytes obtained from the spleen, bone marrow, lymph node, and peritoneum of mice at various intervals

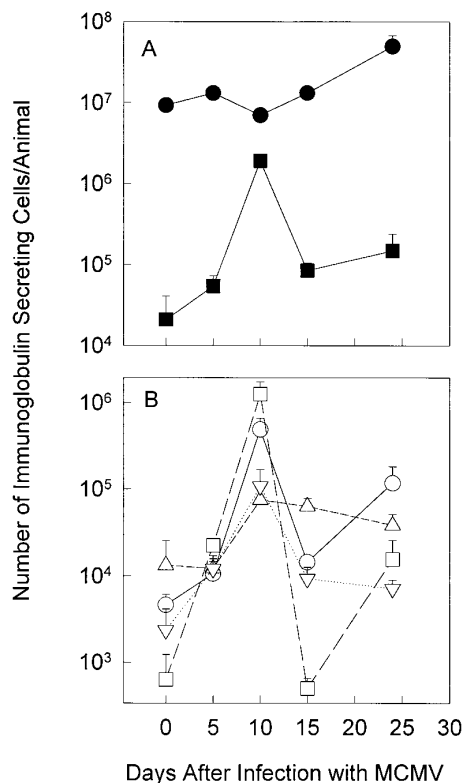


FIG. 3. Enumeration of antibody producing cells by ELISPOT assay of Cx/BK spleen cells. The total number of lymphoid cells producing isotype-specific and subclass-specific antibody were obtained by adding individual numbers measured in the spleen, bone marrow, mesenteric lymph node, and peritoneum. Numbers shown are the average + 1 SD for three mice/group for each time point. (A) IgM (●) and IgG (■) and (B) IgG subclasses: IgG₁ (○), IgG_{2a} (□), IgG_{2b} (△), and IgG₃ (▽). Data shown are representative of two separate experiments with three mice/group for each time point.

following infection. No increase in the number of IgM-producing cells was apparent during the first 15 days after infection, although a 10-fold increase was noted in a single experiment at day 24 (Fig. 3). In contrast, the total number of IgG-secreting cells was increased at 5 days after infection, peaked at day 10, and decreased thereafter. At day 24, the numbers were still higher than those for uninfected mice. Analyses of individual IgG subclasses indicated that maximal changes occurred at day 10 postinfection, consistent with flow cytometric analyses. At that time, the most pronounced increases were seen in the number of IgG_{2a}-producing cells (2000-fold), followed by IgG₁ (100-fold), IgG₃ (50-fold), and IgG_{2b} (10-fold) (Fig. 3).

Antibody specificity of ig-secreting cells

The reactivity of the antibody in sera from MCMV-infected mice with tissues from uninfected syngeneic mice (Bartholomaeus *et al.*, 1988) is believed to be associated with the production of autoantibodies as a consequence of infection. We therefore investigated whether B cell activation induced by MCMV was polyclonal in

nature and if this phenomenon was responsible for autoantibody production by testing the antibody specificity of Ig-producing cells. At 10 days after infection, the number of IgM-producing cells was relatively unchanged, and the antibody they produced had minimal reactivity with any of a series of autoantigens (DNA, myosin, and actin), conventional antigens (ovalbumin and TNP), or the unrelated viral antigen (HIV-1 gp 120) (Figs. 3 and 4). In contrast, the number of IgG-producing cells was increased by about 130-fold (Fig. 4; see also Fig. 3), and the IgG produced by B cells from virus-infected mice displayed a broad range of reactivities with the antigens tested. The number of B cells secreting IgG antibodies against TNP was increased 175-fold; against ssDNA, 180-fold; against myosin, 95-fold; against ovalbumin, 110-fold; against actin, 70-fold; and against HIV gp 120, about 40-fold over the numbers of cells with these specificities from uninfected mice (Fig. 4). Thus, the B cell activation induced by MCMV infection was truly polyclonal and resulted in the production of IgG antibodies against a broad spectrum of conventional, self, and unrelated viral antigens.

Antibody specificity of sera from CD40-deficient mice

MCMV-induced B cell activation was not dependent on the presence of CD4⁺ T cells (Figs. 1B and 2B). This suggested the possibility that the production of antibodies reactive with self, conventional, and unrelated viral antigens may not require signaling via CD40–CD40L. To test this possibility, the reactivity of sera from MCMV-infected CD40-deficient mice with ovalbumin, myosin, and TNP was examined. Ten days after infection, IgG

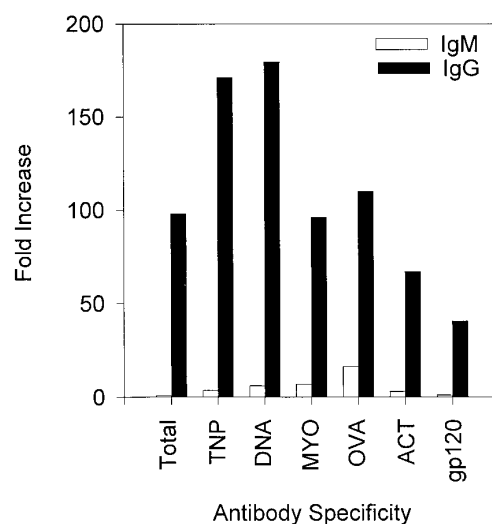


FIG. 4. Antibody specificity of Cx/BK Ig-secreting cells. The fold increases in the numbers of IgM (□)- and IgG (■)-producing cells with reactivity to unrelated antigens was determined as described. Antibodies of the IgG isotype displayed reactivity with a panel of autoantigens (ssDNA, actin, and myosin), conventional antigens (TNP and ovalbumin), and HIV-1 gp 120. Data shown are representative of two separate experiments with three mice/group for each time point.

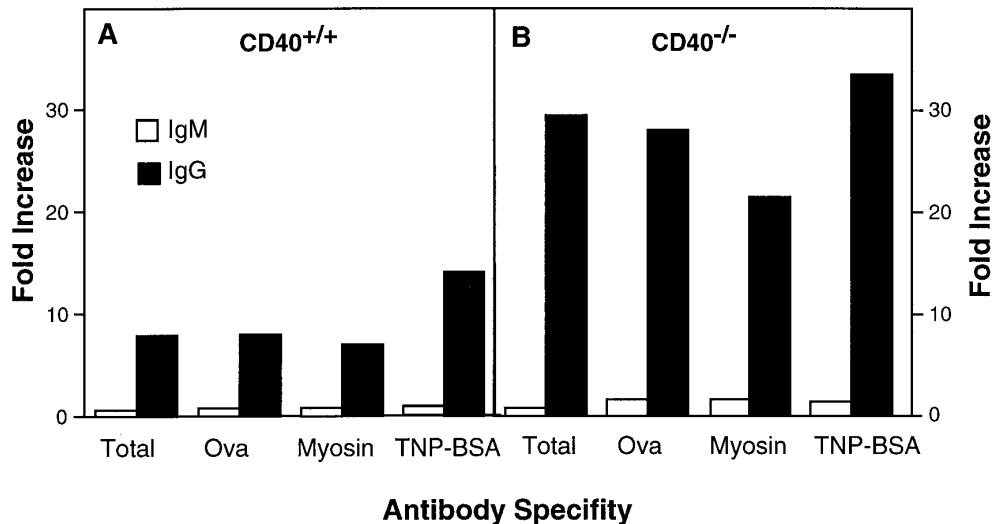


FIG. 5. Antibody specificity of sera from CD40-deficient B6 \times 129 mice. The fold increases in levels of serum Ig in MCMV-infected CD40^{+/+} (A) and CD40^{-/-} (B) mice reactive with ovalbumin, myosin, and TNP above levels in uninfected control B6 \times 129 mice were determined. Results are representative of two separate experiments with five mice/group.

isotype antibodies reactive with all three antigens were increased between 20- and 30-fold compared with antibody levels in sera from uninfected CD40-deficient mice (Fig. 5). The increases were between 2- and 3-fold higher than increases seen in similarly infected wild-type mice. The extremely low levels of circulating IgG in sera of uninfected mutant mice most likely contributed to the massive increase in the levels of total IgG as well as IgG crossreactive with self and conventional antigens. MCMV-induced polyclonal B cell activation was thus independent of CD40/CD40L interaction.

Analysis of cytokine mRNA expression by RT-PCR

Although the polyclonal B cell activation in MCMV-infected mice was independent of CD4⁺ T-cell-mediated help, the process could nevertheless be driven by cytokines produced by cell populations other than this T cell subset. We therefore analyzed the induction of mRNA transcripts for a number of cytokines in the spleen using RT-PCR. By this procedure, it was found that the levels of mRNA for IL-2 and the p40 chain of IL-12 did not change significantly compared with uninfected controls during the first 10 days of infection but were below the levels expressed by uninfected animals at day 14 (Fig. 6a). The mRNA transcripts for TNF- α were increased only by 1.2- to 2-fold over the entire course of infection analyzed. The most striking changes in the levels of mRNA transcripts, however, were observed for IL-4, IL-6, IL-10, and IFN- γ , cytokines known to influence B cell proliferation, activation, and Ig synthesis. Among these, only IL-4 mRNA expression was severely depressed during the first few days after infection, and especially at day 5 (down by 500,000-fold) (Fig. 6b). At days 10 and 14, however, the levels of IL-4 increased by 9- and 4-fold, respectively, over basal levels. In contrast to IL-4, levels of IL-6 and

IL-10 mRNA expression were increased by several orders of magnitude at 3 day p.i. and remained high through day 10 for IL-6 and day 7 for IL-10, returning to background levels thereafter (Figs. 6a and 6b). IFN- γ mRNA transcripts were also increased over the course of infection with kinetics similar to IL-6, although the fold increases were not as dramatic. No changes above the very low background levels in mRNA transcripts were noted for IL-5 and IL-13, two other cytokines that could potentially affect B cell antibody responses (data not shown).

Quantitation of cytokines by ELISA and ELISpot assay

Measurements of cytokine expression at the protein level were performed to determine whether increases in the mRNA transcripts were paralleled by increased protein products. First, supernatants from mitogen-stimulated and unstimulated splenocyte cultures were used to measure cytokine levels by ELISA. Second, splenocytes obtained from uninfected and virus-infected mice were used *ex vivo* in an ELISpot assay to determine whether the changes in cytokine mRNA expression levels were related to increases in numbers of cytokine-producing cells following infection with MCMV.

IL-2 was not detected by ELISA in unstimulated (spontaneous production) or LPS-stimulated cultures of spleen cells from uninfected or MCMV-infected mice. Stimulation with Con A resulted in IL-2 production in splenocyte cultures established from uninfected mice or from mice infected for 3 days (Fig. 7A), but this response was lacking in cultures of spleen cells obtained from mice 5 and 7 days after infection. In the case of IFN- γ , no spontaneous production was noted even after infection, but large amounts of this cytokine were detected in cultures treated with Con A or LPS. IFN- γ production in

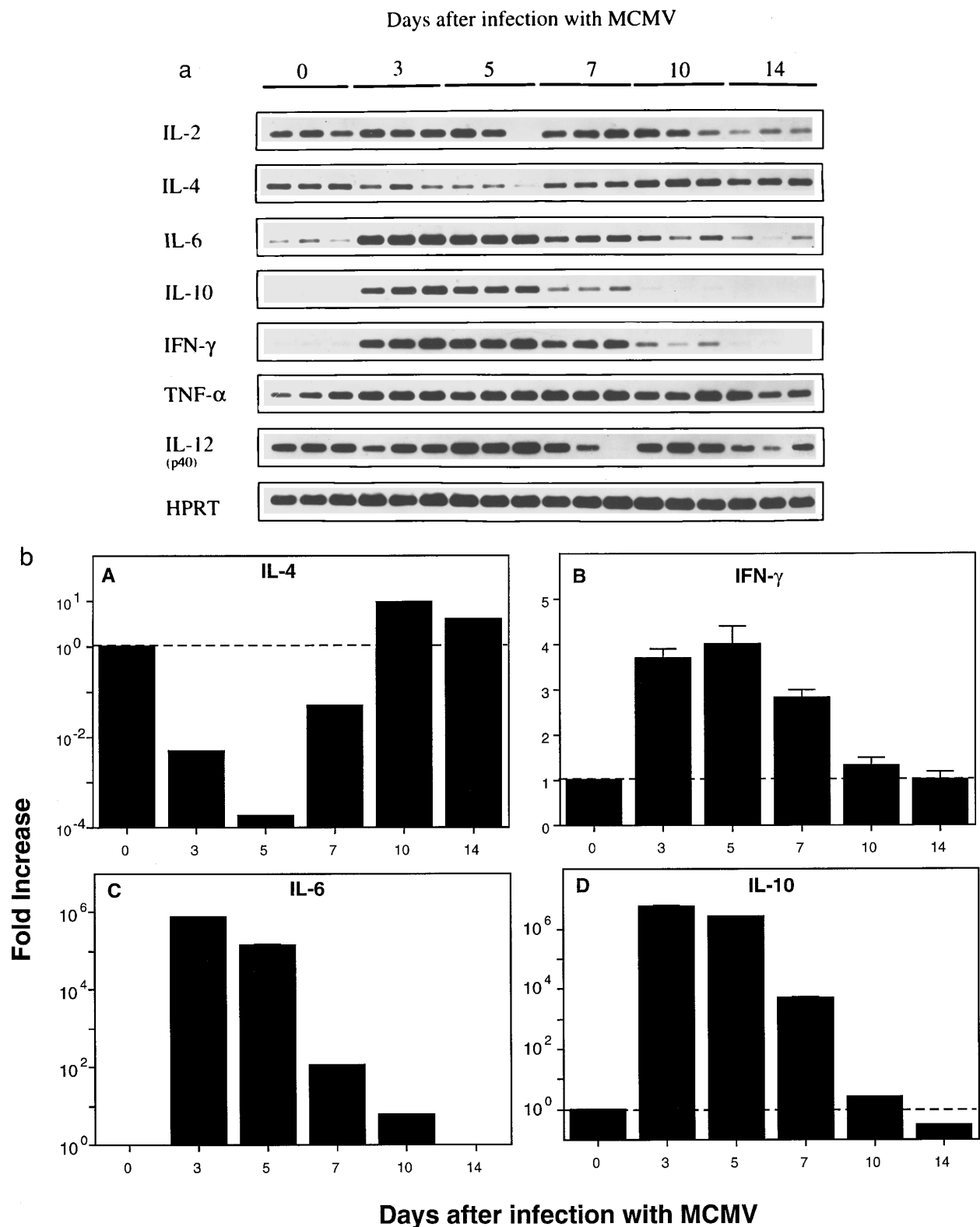


FIG. 6. (a) Kinetics of cytokine mRNA expression in MCMV-infected C×BK mice. mRNA isolated from spleens of uninfected (day 0) or MCMV-infected (days 3, 5, 7, 10, and 14) mice was reverse transcribed, the cDNA was amplified using appropriate cytokine primer pairs, and products were detected after electrophoresis. Southern blotting and hybridization with relevant probes were as described. The resultant bands show areas of specific probe hybridization. For each day, mRNA samples from spleens from three animals were used. (b) Fold increase/decrease of cytokine mRNA expression in spleens of MCMV-infected mice over control, uninfected mice. The band intensities were quantitated by densitometry. Values are expressed relative to uninfected controls (day 0), which are assigned an arbitrary value of 1. Data represent the mean and the SD from the spleens of three individual mice. Results are representative of two separate experiments with three mice/group for each time point.

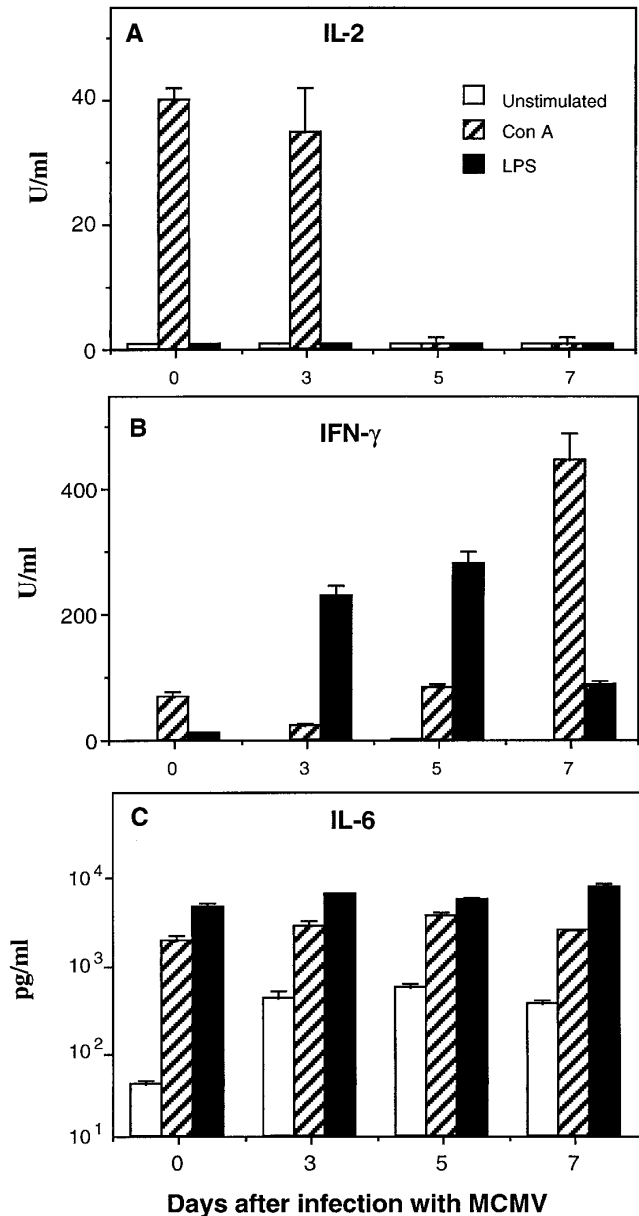


FIG. 7. Quantitation of cytokines (protein) by ELISA. The presence of (A) IL-2, (B) IFN- γ , and (C) IL-6 in unstimulated, Con A-stimulated, or LPS-stimulated splenocyte cultures of C \times BK mice was determined. Data shown are representative of two separate experiments using splenocytes from individual mice in triplicate cultures for each restimulation condition.

response to LPS peaked between days 3 and 5 postinfection, whereas the Con A response peaked at day 7 (Fig. 7b). This may be a reflection of different cell populations producing this cytokine at different times after infection. In contrast to IL-2 and IFN- γ , spontaneous IL-6 production was detected in splenocyte cultures from uninfected mice, and this increased by more than 10-fold at day 3 p.i., over 13-fold at day 5, and remained increased, about 9-fold over endogenous levels, at day 7 p.i. Stimulation with Con A and LPS enhanced IL-6 production by several orders of magnitude (Fig. 7C).

Under conditions of stimulation with Con A or LPS or no stimulation, neither IL-4 nor IL-10 was detected in cultures by ELISA.

The total numbers of cells producing IL-2, IL-4, IL-6, and IFN- γ were enumerated by the ELISpot assay (Fig. 8). While the number of IL-2 producing cells actually decreased over the course of infection, the numbers of IL-6 and IFN- γ -producing cells were clearly increased, consistent with changes that correlated with maximal production of these cytokines detected by ELISA (Fig. 8). Although IL-4-producing cells were detected by the ELISpot assay, the numbers did not change during the course of infection. Failure to detect IL-4 by ELISA may be due to an inability of the assay to detect very low levels of the cytokine. The reason for the unusually low numbers of IL-2-producing cells at day 5 is not clear, but it was a reproducible finding.

Response of mice deficient in IL-6 or IFN- γ to MCMV

The two proinflammatory cytokines IL-6 and IFN- γ profoundly influence B cell physiology and can be produced by non-T cells. Both cytokines, and in particular IL-6, were produced in large amounts by splenocytes from MCMV-infected mice. In order to determine whether either of these cytokines contributed to virus-induced polyclonal B cell activation, mice deficient in IL-6 or IFN- γ and appropriate controls were inoculated with MCMV. Flow cytometric analysis of splenocytes 10 days after infection confirmed that the B cell activation responses in IFN- $\gamma^{-/-}$ and IFN- $\gamma^{+/+}$ mice were similar except that the proportion of IgG_{2a}-expressing cells was decreased in the mutant mice by twofold, and a concomitant increase in IgG₁ expression was noted (data not shown). In contrast, similar analysis of splenocytes from

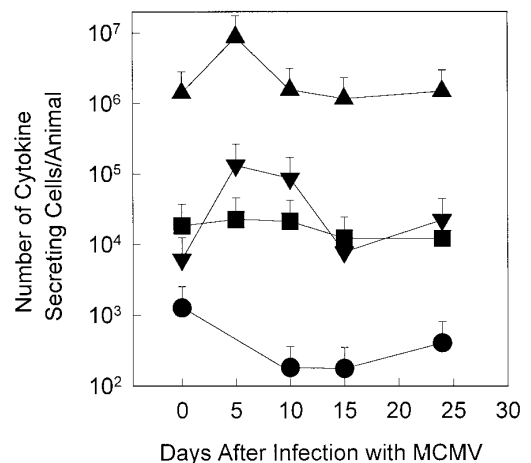


FIG. 8. Enumeration of cytokine-producing cells by ELISpot assays of C \times BK mice. Data shown are from one of two experiments with similar outcomes using three mice for each time point. The numbers of cells producing IL-2 (●), IFN- γ (▼), IL-4 (■), and IL-6 (▲) were determined in the spleens of uninfected (day 0) and MCMV-infected (days 5, 10, 15, and 24) mice.

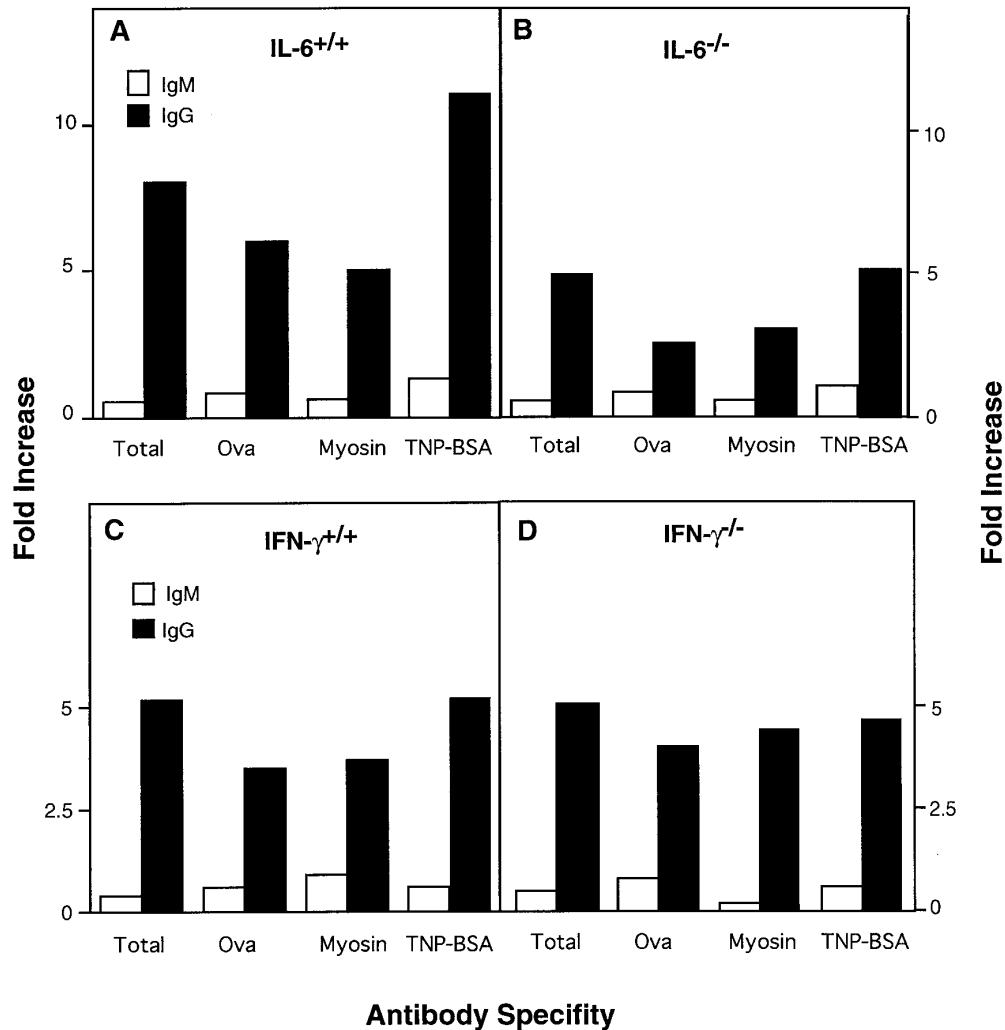


FIG. 9. Antibody specificity of sera from IL-6-deficient and IFN- γ -deficient mice 10 days after infection with MCMV. The fold increases over normal in levels of serum Ig in IL-6^{+/+} (A), IL-6^{-/-} mice (B6 \times 129 background) (B), IFN- γ ^{+/+} (C), and IFN- γ ^{-/-} (D) mice (B6 background) reactive with ovalbumin, myosin, and TNP were determined. Data shown are representative of two separate experiments using five mice for each group. ELISAs were carried out in triplicate for individual serum samples against each antigen tested. The SEM were less than 5% and have been omitted for clarity.

IL-6^{-/-} mice indicated that B cell activation was not as profound as that seen in the control IL-6^{+/+} mice (data not shown). While the levels of CD45R, surface IgM, and Ig κ expression in MCMV-infected IL-6^{+/+} mice were reduced dramatically (comparable to Fig. 1), those in IL-6^{-/-} mice were not (data not shown).

The reactivity of serum IgG from MCMV-infected IL-6^{+/+} mice with ovalbumin, myosin, and TNP was increased by 5- to 10-fold over that of uninfected IL-6^{+/+} mice (Fig. 9); however, sera from virus-infected IL-6^{-/-} mice had lower levels of ovalbumin-, myosin-, and TNP-specific IgG. Compared with MCMV-infected IL-6^{+/+} mice, the fold increases of the crossreactive antibodies in mutant mice were about 50% less, and fold increases in total IgG were 1.5-fold lower. The levels of ovalbumin-, myosin-, and TNP-crossreactive IgG and IgM antibodies in uninfected mutant and wild-type mice were similar.

Deficiency in IFN- γ did not affect the capacity of B cells from MCMV-infected mice to produce IgG antibod-

ies reactive with ovalbumin, myosin, and TNP (Fig. 9). The only obvious difference was that in IFN- γ ^{-/-} mice, serum IgG_{2a} levels were reduced by threefold with a concomitant twofold increase in IgG, levels compared with IFN- γ ^{+/+} mice (data not shown).

Histopathology

Studies of tissues obtained at autopsy of infected mice showed striking germinal center expansion in spleens and lymph nodes that was maximal by day 10 after infection (Fig. 10) but had resolved almost entirely by day 21. In contrast, tissues of infected mice deficient in CD4⁺ T cells as a result of treatment with anti-CD4 mAb, absent MHC class II expression or the nude defect, were strikingly different in being devoid of germinal centers. The absence of germinal centers in CD4-deficient mice can most likely be explained by the absence of T cells expressing CD40L, which is required for germinal center

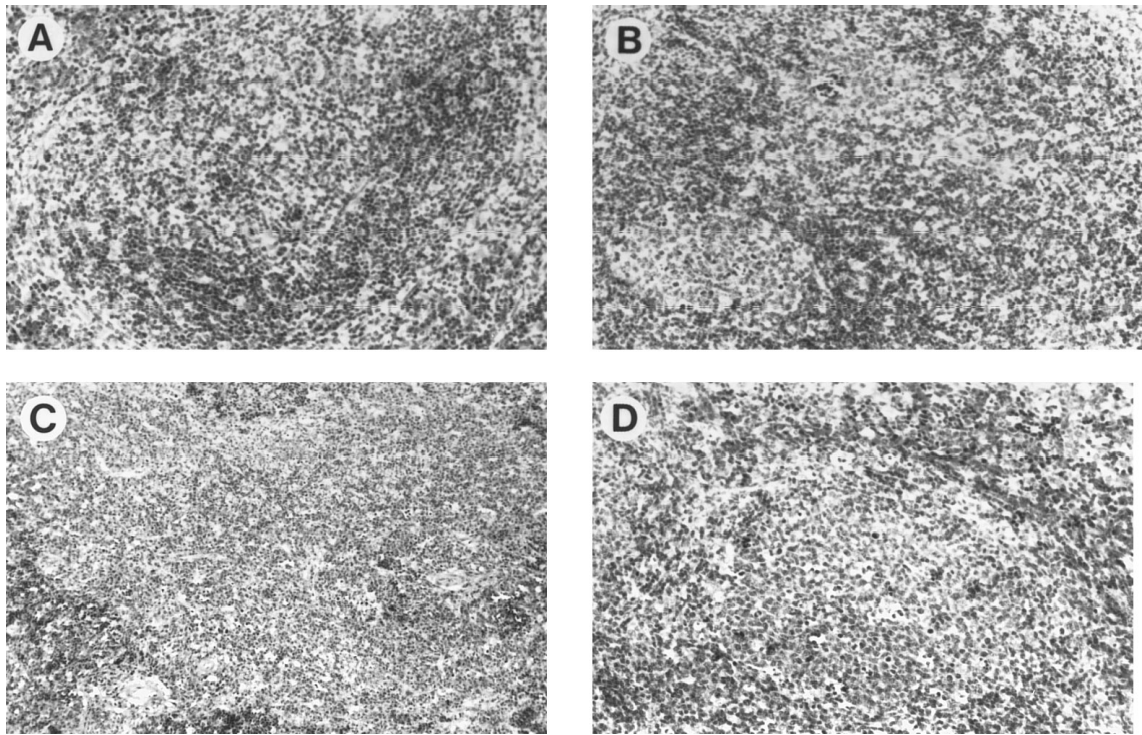


FIG. 10. All photomicrographs are of sections of the splenic white pulp stained with hematoxylin and eosin at a magnification of $\times 200$. (A) MHC class II knockout uninfected mouse spleen with minimal activity in the white pulp; the follicle consists mainly of small lymphocytes, the dark-staining cells of the mantle zone. There are no germinal centers present in any follicle. (B) Wild-type mouse also uninfected but with a typical small germinal center within the follicle (lower left) indicating the moderate activity often seen in normal spleen. (C) MHC class II knockout infected with MCMV. The follicle is about the same size as in the uninfected spleen (A), but there is a loss of mantle zone cells and a continued absence of germinal centers. (D) Wild-type mouse infected with MCMV with a very large, active germinal center within the follicle occupying the entire central portion of the photomicrograph.

formation (Durie *et al.*, 1994; Kawabe *et al.*, 1994). Flow cytometric analyses revealed comparable B cell changes in spleens of infected mice with and without $CD4^+$ T cells either having or lacking germinal centers, respectively.

DISCUSSION

Data presented here establish that MCMV induces polyclonal B cell activation during the acute phase of primary infection. The antibodies produced had broad crossreactivity with a panel of autoantigens, conventional antigens, and the unrelated HIV-1 gp 120. These results are consistent with previous studies of the MCMV model (Price *et al.*, 1993) and may be pertinent to the occurrence of autoreactive antibodies in humans following infection with HCMV (Hutt-Fletcher *et al.*, 1983). The culturing of live or UV-inactivated HCMV with human peripheral blood B cells has been shown to induce polyclonal B cell activation and Ig synthesis, suggesting that some component(s) of the virus can either directly or indirectly activate B cells and that this process is independent of $CD4^+$ T cells. In the current studies, infection with MCMV-induced B cell activation in two different models of $CD4^+$ T-cell-deficient mice and in *nu/nu* mice, which lack functional $CD4^+$ and $CD8^+$ T cells. This

finding differs from a previous study (Price *et al.*, 1993) in which the activation of B cells following infection with MCMV was found to be dependent on $CD4^+$ T cells. The differences with respect to the requirement for helper functions of $CD4^+$ T cells in our study and the previous report are not clear. They cannot be attributed to differences in virus, as both studies used the Smith strain of MCMV passaged in salivary gland and both studies examined mice depleted of $CD4^+$ T cells with GK1.5 mAb. Furthermore, in both studies, the phenomenon of B cell activation was observed in all strains of mice tested, irrespective of their genetic resistance to infection and disease.

Depressed IL-2 secretion and IL-2 receptor expression by spleen cells in response to Con A stimulation are two other known consequences of infection with MCMV (Nicholas *et al.*, 1987; Blackett and Mims, 1988). From our studies, it appears that the reduced levels of IL-2 can be ascribed to reduced IL-2 transcription. The capacity of spleen cells to produce IL-2 in response to Con A, evident at 3 days after infection, was lost by day 5. This loss in the ability to produce IL-2 did not correlate with virus infectivity levels in the spleen, as viral titers were comparably high on both day 3 and day 5 after infection (data not shown). Further, suppression of this response is

known to last for at least 14–18 days p.i. (Nicholas *et al.*, 1987), whereas virus in the spleen was cleared by day 10 (data not shown). Thus, while active production of virus per se does not appear to inhibit the capacity of spleen cells to produce IL-2 in response to Con A stimulation, it is possible that some viral protein(s) or virus-induced factor(s), produced as a consequence of virus replication possibly in a distant organ, e.g., the salivary gland, is responsible for the suppression of IL-2 production. In this respect, it is interesting to note that the HIV-1-soluble gp 120 causes a similar suppression of IL-2 production and IL-2 receptor expression (Oyaizu *et al.*, 1990; Bowen *et al.*, 1992). The failure of T cells from MCMV-infected mice to produce IL-2 while maintaining the ability to produce IFN- γ resembles the cytokine profile of anergic Th1-type cells; however, the "anergy" associated with MCMV is observed only during the acute phase of infection.

The broad reactivity of antibodies from virally infected animals was confined to the IgG isotype. By flow cytometric analysis, the most profound changes for surface expression of the IgG subclasses were the striking increases in IgG_{2a} and IgG_{2b}. Further, by ELISpot assay, maximal increases were noted in the numbers of IgG_{2a}-producing cells. Production of this IgG subclass is strongly stimulated by IFN- γ . We successfully demonstrated, both at the mRNA and at the protein levels, that this cytokine is produced early in infection. The kinetics of IFN- γ expression following stimulation of spleen cells with either LPS or Con A suggested that NK cells are likely to be the predominant source soon after infection and T cells later on (Figs. 7A and 7B). LPS is known to induce macrophages to produce IL-12, which, in turn, is highly efficient at inducing NK cells to produce IFN- γ before virus-specific T cell responses develop (days 3 and 5; Fig. 7B). These results are consistent with earlier studies that demonstrated a peak in serum levels of IFN- γ at 40 h postinfection and a dependency of this early response on IL-12 triggering of IFN- γ production by NK cells (Orange and Biron, 1996; Cousens *et al.*, 1997; Ruzek *et al.*, 1997). Production of IFN- γ by NK cells was found to be facilitated by TNF- α (Orange and Biron, 1996), and early production of both cytokines is suggested to result in macrophage activation as an important component of the innate response to virus infection (Heise and Virgin, 1995). Preferential induction of IFN- γ by Con A at day 7 suggests that T cells may be the primary source of this cytokine at later time points. This is consistent with reports that IFN- γ secretion by CD4⁺ T cells is important for the control of virus replication in the salivary glands (Jonjic *et al.*, 1989; Koszinowski *et al.*, 1990). Virus seeds this organ later in infection and persists longer than in other organs. Interestingly, and quite predictably, in mice in which this cytokine was neutralized *in vivo* (data not shown) or in IFN- γ -deficient mice, the frequency of IgG_{2a}-expressing cells was severely reduced, but there was a concomitant increase in the

frequency of surface IgG₃- and, in particular, IgG₁-expression cells (data not shown). Thus, the absence of IFN- γ did not abolish B cell activation by the criteria used in these studies but did influence IgG switching preference. It is likely that in the absence of IFN- γ , enhanced IL-4 production results that contributes to the shift from IgG_{2a} to IgG₁ expression. In MCMV-infected mice, mRNA transcripts for IL-4 were severely depressed, and no IL-4 could be detected under the conditions of restimulation used in our studies. The lack of detection of IL-4 by ELISA may have been the result of lower levels of sensitivity of this assay. Using the ELISpot assay, IL-4-producing cells were detected, but the numbers did not change significantly over the entire course of infection.

That IL-2 expression (at the protein level) is reduced during the acute phase of infection with MCMV has been reported previously (Nicholas *et al.*, 1987; Blackett and Mims, 1988). The absence of a correlation between IL-2 mRNA (RT-PCR) and protein (ELISA and ELISpot) expression may be due to posttranscriptional regulation of cytokine gene expression *in vivo*.

While mRNA for IL-10 was induced at very high levels in the spleen following infection with MCMV, no protein could be detected following restimulation of splenocytes *in vitro*.

The rapid induction of mRNA for IL-6, a known B cell stimulatory factor, was associated with increased spontaneous production of the cytokine over the course of infection with MCMV. Since unregulated and constitutive production of IL-6 has been associated with polyclonal B cell activation leading to hypergammaglobulinemia and autoantibody production in certain tumor-bearing patients (Hirano *et al.*, 1987) and in individuals infected with HIV-1 (Breen *et al.*, 1990), we speculated that this factor may be a major driving force for the polyclonal B cell activation in MCMV-infected mice. Furthermore, IL-6-transgenic B lineage cells that develop in SCID or Rag-2-deficient mice after transplantation give rise to nearly normal levels of some subclasses of IgG and of IgA (Oka *et al.*, 1995). Indeed, in IL-6-deficient mice infected with MCMV, the level of B cell activation was not as dramatic as in IL-6^{+/+} mice. Furthermore, antibodies reactive with ovalbumin, myosin, and TNP were reduced by almost 50% in mutant mice compared with virus-infected wild-type mice. The serum IgG levels in mutant mice were also lower than those in wild-type mice. Taken together, these results are consistent with findings that the production of autoreactive antibodies in systemic autoimmunity is due to polyclonal activation and not the specific stimulation of autoreactive clones (Klinman and Steinberg, 1987); however, unlike HIV-1 infections in humans, IL-6 production in MCMV infection is a transient event.

Another finding that needs to be addressed experimentally is the relevance of the increased frequency of NK1.1⁺ cells to B cell activation. The kinetics of increased NK1.1 antigen expression on splenocytes closely paral-

leled the kinetics of B cell activation. The expression of the NK cell marker did not correlate with the cytolytic activity of NK cells (data not shown) in MCMV-infected mice, because the killing activity declined to nearly background levels (as in uninfected mice) when NK1.1 expression was maximal. Indeed, NK cells—known to produce IFN- γ early in infection—have been shown to induce activated murine B cells to secrete Ig in response to a T-cell-independent antigen (Snapper *et al.*, 1993).

Virus persistence and the establishment of latency in the host are classic sequelae of infection with herpesviruses, which include CMV. Although mechanisms underlying the ability of CMV to establish latency are poorly defined, virus-induced immunosuppression during the acute phase of infection is believed to be contributory (Banks and Rouse, 1992). Several other strategies, of which polyclonal B cell activation could be one, may be employed by the virus to establish latency.

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